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Rev-Dependent Expression of Human Immunodeficiency Virus Type 1 gp160 in *Drosophila melanogaster* Cells

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Expression of the human immunodeficiency virus (HIV) structural proteins in mammalian cells is regulated posttranscriptionally by the viral Rev protein. Rev has been shown to *trans*-activate expression by relieving the nuclear sequestration of RNAs containing viral *gag* or *env* coding regions. We have studied the effects of Rev on expression of the HIV type 1 *env* gene in *Drosophila melanogaster* cells. We demonstrated that synthesis of the gp160 envelope protein was fully Rev dependent; that is, gp160 was produced only when Rev function was coexpressed in the cell. Analysis of total cellular RNA indicated that Rev did not significantly affect the overall levels of gp160 RNA production. Instead, mRNA encoding gp160 was found in the cytoplasm only in cells expressing Rev, whereas in cells lacking Rev, this RNA was present only in the nucleus. Furthermore, comparison of these results with the previously demonstrated Rev-independent expression of gp120 envelope protein with this system indicated that information contained in the gp41 coding region appears to be critical to the selective nuclear retention of gp160 transcripts in the absence of Rev. Our results clearly demonstrate that the mechanism of Rev action is conserved in the insect cell system, and thus, Rev must function via cellular machinery common to most, if not all, higher cell systems.

The human immunodeficiency virus type 1 (HIV-1) encodes two regulatory proteins, Tat and Rev, which govern viral gene expression and are essential for virus replication (11, 16, 18, 44). Viral proteins are expressed from a set of RNAs generated from a single primary transcript, with structural proteins being expressed from unspliced (*gag*) or singly spliced (*env*) mRNAs and regulatory proteins being expressed from smaller multiply spliced mRNAs (2, 36, 42). Whereas the Tat protein increases the expression of both structural and regulatory proteins of the virus, the Rev protein selectively increases the synthesis of structural proteins (reviewed in reference 8). Both transcriptional and posttranscriptional mechanisms have been proposed for Tat (reviewed in reference 24). In contrast, the Rev protein acts posttranscriptionally (15, 16, 20, 26, 29, 44).

Several studies have indicated that Rev enhances structural gene expression by allowing the cytoplasmic accumulation of unspliced *gag* or singly spliced *env* mRNAs (1, 15, 17, 21, 30). In the absence of Rev, *gag* and *env* mRNAs appear to be retained in the nucleus by a mechanism that may involve negative elements in the mRNA (12, 15, 21, 40) and the cellular splicing machinery (5). Rev is thought to rescue these mRNAs by perturbing the interaction of messages with the splicing machinery or by directly promoting their interaction with transport factors (15, 17, 30, 40). Recent data suggest that Rev action is restricted to mRNAs which maintain suboptimal recognition by the cellular splicing machinery (5). The ability of Rev to facilitate mRNA export into the cytoplasm requires a *cis*-acting Rev response element (RRE) in the RNA, which is located in the *env* coding region (20, 21, 30, 40). Extensive mutational analyses have delineated a complex secondary structure for the RRE and have identified regions within the structure that are crucial for Rev function (6, 13, 30, 31, 37). Rev protein has been shown to specifically recognize the RRE in vitro (10, 22, 45), and mutations that reduce this binding also impair Rev response in vivo (31, 37).

The precise mechanism of Rev function remains unknown. Rev is targeted to the nucleus, where it localizes primarily in the nucleolus (9, 17, 28, 38). This localization is thought to be important for Rev function, since mutations that eliminate nucleolar localization also reduce Rev function (7, 28, 38). Rev has been shown to function in a number of different cell types, including human (1, 12, 17, 40, 44), monkey (12, 15, 16, 21, 26, 29, 41), and hamster (37, 40) cell lines. This suggests that the mechanism of Rev function is conserved in primates and in at least one other mammalian cell type. Rev regulation has not been demonstrated before in any nonmammalian system.

We have examined the expression of the HIV-1 *env* gene in *Drosophila melanogaster* Schneider 2 cells. We found that gp160 expression was dependent on coexpression of the *rev* gene product in these cells. Analysis of RNA localization indicated that the mechanism of Rev action on *env* mRNA which occurs in mammalian systems is conserved in *Drosophila* cells. These results are discussed in light of the proposed mechanisms for Rev function.

MATERIALS AND METHODS

Vector construction. Plasmid constructs were generated by standard cloning techniques (32). All plasmid constructs were analyzed for correct junction sequences by dideoxy sequencing (Sequenase Version 2.0; U.S. Biochemical Corp.). Plasmid pMt160Δ32 was derived from pMtBH10 (M. Ivey-Hoyle et al., Proc. Natl. Acad. Sci. USA, in press) by replacing the *Pvu*II-*Sac*I *env* fragment with the *Pvu*II-*Xho*I *env* fragment of HIV-1 BH10 sequences (36) and a synthetic *Xho*I-*Sac*I linker. The resulting construct (Fig. 1) contains an expression unit consisting of the inducible *Drosophila* metallothionein (Mt) promoter, the beginning of the human tissue plasminogen activator (tPA) gene fused in frame with gp160 coding sequences (nucleotides 5986 to 8479 of reference 36), and finally the simian virus 40 (SV40) early

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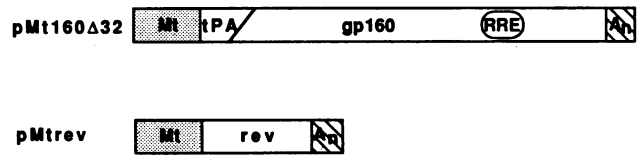

FIG. 1. *Drosophila* expression vectors. The DNA constructs used to produce HIV-1 BH10 proteins in *Drosophila* cells are shown. The open reading frames (open boxes) are preceded by the inducible *Drosophila* Mt promoter (Mt, dotted box) and followed by the SV40 early polyadenylation sequence (A_n, hatched box). The expression units shown are present on a pBR322-based plasmid. For expression of gp160 (pMt160Δ32), the *env* signal sequence and first 31 amino acids of the mature viral protein were replaced with the signal sequence of the human tPA gene (tPA). This in-frame fusion is represented by a slash mark. pMtRev contains a copy of the *rev* cDNA and encodes authentic Rev protein.

polyadenylation signal. The gene fusion creates an open reading frame encoding the first 36 amino acids of human tPA (signal sequence) fused to 795 amino acids of gp160 beginning with amino acid 32 (Asp) of the mature viral molecule and ending at the natural gp160 stop codon. Cleavage of the tPA signal sequence from an identical tPA fusion with gp120 (pMtBH10) resulted in a secreted gp120 molecule whose N-terminal 31 amino acids were replaced by four amino acids from tPA (Ivey-Hoyle et al., in press).

pMtRev (Fig. 1) was constructed by inserting an *Xba*I-*Xho*I fragment encompassing the Rev cDNA from pH3art (40), (kindly provided by W. A. Haseltine, Dana-Farber Cancer Institute) into an *Xba*I-*Xho*I-cleaved *Drosophila* expression vector containing the Mt promoter and SV40 early polyadenylation signal on a pBR322-based plasmid.

Cell culture, transfection, and inductions. *D. melanogaster* Schneider 2 cell culture and generation of stably transfected cell lines by a hygromycin B selection method have been described before (23). A total of 20 μg of plasmid DNA was used in each transfection, including 10 μg of the hygromycin B selection plasmid pCOhygro (23) and 10 μg (total) of pMt160Δ32 and pMtRev, either singly or in combination. Expression from the Mt promoter was induced by the addition of 0.5 mM $CuSO_4$ to the medium.

Protein analysis. Induced or uninduced cells were removed from the medium by centrifugation, and whole-cell lysates were prepared by suspending cells at 10^8/ml in Laemmli sample buffer (27) and incubating at 95°C for 4 min. Culture supernatants were also prepared by adding sample buffer and incubating at 95°C. For analysis of envelope proteins, 7 μl of whole-cell lysates (7×10^5 cells) or 15 μl of culture supernatant samples was separated on a 10% polyacrylamide–sodium dodecyl sulfate (SDS) gel and transferred electrophoretically to nitrocellulose for Western immunoblot analysis (19). Envelope proteins were detected by using rabbit antisera to purified HIV-1 BH10 gp120 produced in *Drosophila* cells, followed by ^{125}I-labeled protein A (New England Nuclear). The gp120 envelope standard shown in Western blots was also expressed in *Drosophila* cells (Ivey-Hoyle et al., in press). The gp160 envelope standard was expressed from a baculovirus vector and was kindly supplied by Dirk Gheysen (SmithKline Beecham, Rixensart, Belgium). For analysis of Rev proteins, 20 μl of whole-cell lysates (2×10^6 cells) was separated on a 15% polyacrylamide–SDS gel and transferred to nitrocellulose. Rev protein was detected by using a Rev-specific rabbit polyclonal antibody kindly provided by W. A. Haseltine (Dana-Farber Cancer Institute).

Molecular mass markers were prestained protein standards from Bethesda Research Laboratories as follows: myosin, 210 kDa; phosphorylase B, 107 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 29 kDa; β-lactoglobin, 18 kDa; and lysozyme, 14 kDa.

RNA preparation. Total RNA was prepared from 2.5×10^6 cells as described previously (34) except that 20 mM ribonucleoside-vanadyl complexes (Sigma Chemical Co.) were added to the lysis buffer. Nuclear and cytoplasmic RNA were prepared by a Nonidet P-40 (NP-40) lysis method. Cells (10^7) were washed once with ice-cold phosphate-buffered saline. The cell pellet was suspended in 0.6 ml of NP-40 lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 8.6], 0.5% NP-40, 4 mM ribonucleoside-vanadyl complexes) and spun immediately at $1,000 \times g$ for 5 min at 4°C. The supernatant (0.4 ml) was transferred to an Eppendorf tube for isolation of cytoplasmic RNA. An additional 11 μl of ribonucleoside-vanadyl complexes was added, and the sample was spun for 5 min at $8,000 \times g$ (10,000 rpm) in an Eppendorf microfuge. The supernatant (0.4 ml) was transferred to a new tube containing 0.4 ml of 2 × PK buffer (0.2 M Tris hydrochloride [pH 7.5], 0.44 M NaCl, 2% SDS, 25 mM EDTA) and 8 μl of 20-mg/ml proteinase K (Sigma Chemical Co.). After 1 h at 37°C, the sample was extracted with 0.6 ml of phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. The pellet from the NP-40 lysis and centrifugation step was suspended in 0.3 ml of NP-40 lysis buffer and transferred to an Eppendorf tube for isolation of nuclear RNA. An equal volume of 2× PK buffer and 8 μl of 20-mg/ml proteinase K were added, and the sample was incubated for 1 h at 37°C. The nucleic acid was then isolated as described above for cytoplasmic RNA.

Final nucleic acid pellets from all preparations were dissolved in diethylpyrocarbonate-treated deionized water so that 10 μl would contain the RNA isolated from 1.5×10^6 cells.

Northern (RNA) blot analysis. Total, nuclear, or cytoplasmic RNA from 1.5×10^6 cells (10 μl) was separated on a 1% formaldehyde–agarose gel and blotted to nitrocellulose. A parallel lane containing RNA molecular mass standards (Bethesda Research Laboratories) was stained with ethidium bromide to establish size markers. Hybridization (32) was carried out with restriction fragments labeled by the random primer method (Oligolabeling Kit; Pharmacia).

RNA from an equal number of cells was analyzed from total, nuclear, and cytoplasmic preparations to allow the distribution of messages to be visualized in a quantitative fashion. To ensure that different preparations contained similar amounts of RNA, a probe corresponding to the U2 small nuclear RNA gene (kindly provided by J. Steitz, Yale University) was also used in Northern blots of samples electrophoresed on a 2% formaldehyde–agarose gel. This probe was selected because it would hybridize to RNA present in all three types of RNA preparations.

Analysis of *env*-derived RNAs. cDNA was synthesized from total cytoplasmic RNA with Promega cDNA synthesis reagents and an antisense primer complementary to a 26-nucleotide sequence in the SV40 polyadenylation signal just 5′ to the site of polyadenylation. A second antisense primer within the polyadenylation region and a sense primer identical to *env* sequences 5′ to the expected splice donor site (see Fig. 5A) were used to amplify *env*-derived cDNA by polymerase chain reaction (PCR) technology (Cetus DNA thermal cycler). Antisense primers were designed to hybridize downstream of potential splice acceptor sites. Alternatively, cDNA was synthesized with an oligo(dT) primer with

a polylinker sequence at its 5′ end. PCR amplification was then carried out with a primer identical to the polylinker sequence along with the *env* sense primer described above. PCR-amplified products were purified from 10% polyacrylamide gels by electroelution and sequenced directly with Sequenase Version 2.0 (U.S. Biochemical Corp.). The first approach generated the spliced sequence described in Results (see Fig. 5B, RNA1), and the second approach generated the prematurely terminated *env* sequence described (see Fig. 5B, RNA 2).

RESULTS

Expression of gp160 in *Drosophila* cells requires Rev. We investigated the use of a *D. melanogaster* expression system for the synthesis and study of the HIV-1 BH10 envelope glycoprotein. Previous studies had shown that the extracellular gp120 portion of the *env* gene could be efficiently expressed in *Drosophila* cells (Ivey-Hoyle et al., in press). The construct used replaced the signal sequence and N-terminal 31 amino acids of envelope protein with the signal sequence from the human tPA gene, and this coding information was placed under the transcriptional control of the *Drosophila* Mt promoter. Cell lines stably transfected with this construct expressed 1 to 2 mg of gp120 per liter of culture medium upon metal induction of the Mt promoter.

We made an analogous construct, pMt160Δ32, which encodes the gp160 molecule shown in Fig. 1. This construct also encodes HIV sequences beginning at amino acid 32 of the mature envelope protein, but in this case the sequence continues through the end of the gp160 coding region. In contrast to what was observed with the gp120 construct, *Drosophila* cells stably transfected with pMt160Δ32 failed to produce any detectable gp160 protein upon induction of the Mt promoter (Fig. 2A, cell line A). However, Northern blot analysis of total RNA from this cell line revealed that a full-length transcript was efficiently produced upon metal induction (Fig. 3).

We reasoned that perhaps gp160 expression required the HIV Rev protein, as in mammalian cells. To test this, a construct was made that would express Rev in *Drosophila* cells under the control of the Mt promoter (pMtRev, Fig. 1). Cells were transfected with various amounts of pMtRev, and stable cell lines were selected. Western blot analysis with a peptide-derived antiserum to Rev revealed an appropriately sized protein produced selectively in response to metal induction (Fig. 2B). The levels of Rev protein observed were proportional to the amount of pMtRev DNA used in the transfections.

The Rev expression vector was then cotransfected along with pMt160Δ32 in different ratios to produce stable cell lines containing both transcription units. Upon metal induction, gp160 protein was readily detectable (Fig. 2A, cell lines B, C, and D). Moreover, the level of gp160 protein observed in these stable cell lines increased with an increasing ratio of pMtRev DNA to pMt160Δ32 DNA in the transfections. The results indicate that expression of gp160 protein in *Drosophila* cells is dependent on coexpression of Rev.

Rev-dependent RNA translocation in *Drosophila* cells. To determine the step at which Rev affects *env* gene expression in *Drosophila* cells, RNA was analyzed from cells transfected with either pMt160Δ32 alone or pMt160Δ32 plus pMtRev. Total RNA was isolated from cells before and after induction and subjected to Northern blot analysis with an *env*-specific probe (Fig. 3). Prior to induction, no *env* RNA was detectable in either cell line. This confirmed that transcription from the Mt promoter in these cell lines was tightly controlled. After induction, an appropriately sized envelope RNA (gp160 RNA, approximately 2.8 kb) was synthesized in both cell lines, independent of Rev. In addition, smaller transcripts of about 1.4 to 1.5 kb were also detected with the *env*-specific probe in both cell lines (see below). Thus, the presence of Rev did not appear to have any effect on the synthesis of total cellular *env* RNA.

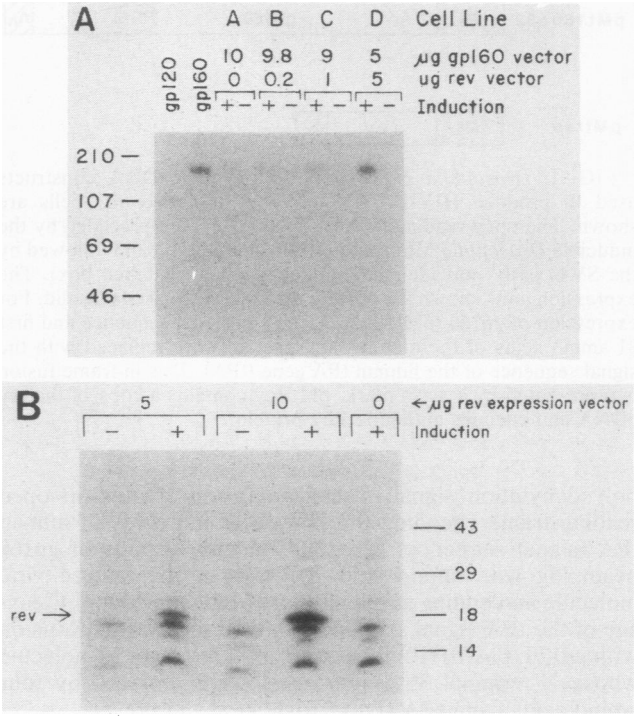

FIG. 2. Western blot analyses of HIV proteins expressed in *Drosophila* cells. (A) Envelope protein expression. Whole-cell lysates from stably transfected cell lines either induced for 5 days (+) or uninduced (−) were electrophoresed on a 10% polyacrylamide–SDS gel and transferred to a nitrocellulose filter. Envelope protein was visualized by Western blot analysis with a rabbit polyclonal antibody to gp120, followed by ^{125}I-protein A. In the first two lanes, gp120 and gp160 protein standards are shown (see text). (B) Rev protein expression. Whole-cell lysates from stably transfected cell lines either induced for 5 days (+) or uninduced (−) were electrophoresed on a 15% polyacrylamide–SDS gel and transferred to a nitrocellulose filter. Rev protein was visualized by Western blot analysis with an anti-Rev antibody, followed by ^{125}I-protein A.

Since an analysis of total cellular RNA did not reveal any significant difference in the *env* RNA synthesized in the presence versus the absence of Rev (Fig. 3), we examined whether Rev affected the nuclear versus cytoplasmic localization of *env* RNA. RNA was isolated from nuclear and cytoplasmic fractions of *Drosophila* cells and subjected to Northern blot analysis (Fig. 4). As with total RNA, nucleus-localized RNA appeared to be the same with and without Rev. In contrast, however, the presence of full-length gp160 RNA in the cytoplasm was dramatically increased in cells expressing Rev over that in cells lacking Rev. Thus, in the absence of Rev, gp160 RNA is present only in the nucleus, and the addition of Rev results in the appearance of gp160 RNA in the cytoplasm. This Rev-dependent shift in nuclear to cytoplasmic localization of gp160 RNA correlates with the Rev dependence of gp160 protein expression in these cells. We point out that the *env*-related RNAs of 1.4 to 1.5 kb were present in the cytoplasm in both Rev$^-$ and Rev$^+$ cell lines. In

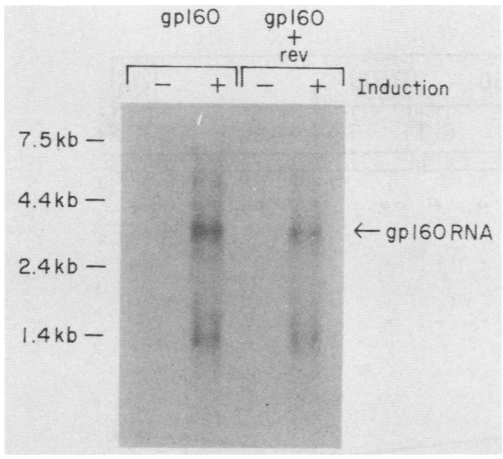

FIG. 3. Northern blot analysis of total RNA with an *env*-specific probe. Total RNA was isolated from cell lines A (gp160) and D (gp160 + Rev), either uninduced (−) or after 5 days of induction (+). The blot was probed with an *env*-specific 935-bp *Stu*I-*Sac*I DNA fragment.

addition to providing an internal control for observing the selective effect of Rev on the translocation of the full-length gp160 mRNA, these smaller RNAs clearly represent *env* transcripts which are not subject to the nuclear retention phenomenon (see Discussion).

In light of recent evidence that splice signal recognition may play a role in the susceptibility of an RNA to Rev regulation (5), it was of interest to determine whether the 1.4- to 1.5-kb *env*-derived RNAs were splicing products of the gp160 RNA. Northern blot analysis with various restriction fragments spanning the pMt160Δ32 construct as probes revealed at least two distinct species of RNA in the 1.4- to 1.5-kb range (Fig. 5A). An RNA of approximately 1.5 kb hybridized to probes from the first third of the *env* gene and from the SV40 polyadenylation region and therefore is presumably a product of splicing. An RNA of approximately 1.4 kb hybridized to probes from the first third of the *env* gene only.

To identify the nature of these RNAs more precisely, cDNA was made from the 1.4- to 1.5-kb RNA-enriched cytoplasmic preparation of cell line A (Fig. 4, lane 5). PCR amplification with appropriate primers was then used to generate DNA fragments corresponding to *env*-derived RNAs. These DNA fragments were gel purified for direct sequencing by the dideoxy method, and the results are shown in Fig. 5B. The sequence of one fragment represented a splice from within *env* to a sequence located in the SV40 polyadenylation region, exactly as predicted by the Northern blot analyses for the 1.5-kb RNA. The sequence of a second fragment represented a premature termination of *env* RNA at a site adjacent to a consensus polyadenylation sequence within *env*, consistent with the hybridization pattern of the 1.4-kb RNA. Thus, we found both prematurely terminated *env* transcripts and products of cryptic splicing in our system.

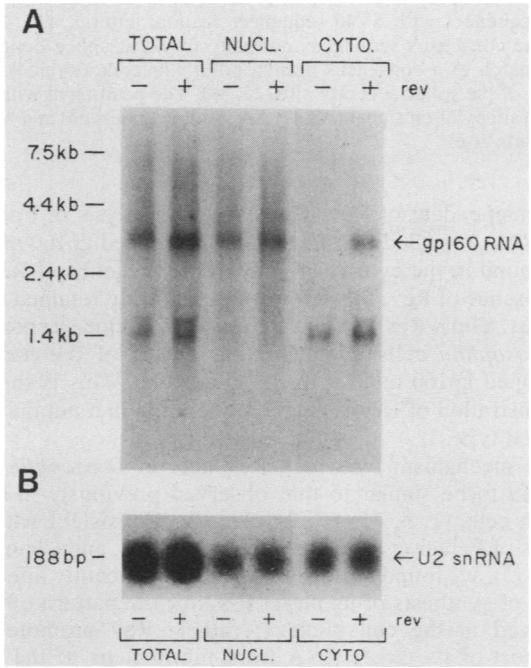

FIG. 4. Northern blot analysis of total, nuclear (NUCL.), and cytoplasmic (CYTO.) RNA. Each lane contains RNA isolated from 1.5×10^6 cells transfected with pMt160Δ32 alone (cell line A, lanes −) or together with pMtRev (cell line D, lanes +) after 5 days of induction. In the top panel, the blot was probed with the same *env*-specific DNA fragment as in Fig. 3. With a control U2 small nuclear RNA gene as the probe (shown at the bottom), it was shown that the various preparations isolated from the two cell lines contained equivalent amounts of RNA.

Processing of gp160 in *Drosophila* cells. The prematurely terminated and spliced forms of *env* RNA detected in the above analysis would be predicted to give rise to a truncated envelope protein with an aberrant C terminus derived from polyadenylated RNA. No such products were detected in cells induced for 5 days (Fig. 2A), perhaps because such aberrant proteins are unstable. In mammalian cells, gp160 is processed to noncovalently associated gp120 (external) and gp41 (transmembrane) proteins, and both products can be readily detected associated with the cell. In contrast, we were unable to detect any processed gp120 associated with the *Drosophila* cells expressing our gp160 construct. One possibility is that the gp160 produced is not processed in *Drosophila* cells. Alternatively, if processed, the resulting gp120 may not remain cell associated.

To look for gp160 and gp120 more thoroughly, cells were harvested every 24 h for 7 days after induction, and both culture medium and cell pellets were analyzed separately for gp120 and gp160 production. The results (Fig. 6) indicate that gp160 protein was detectable in cells even 1 day after induction and increased to a plateau level that persisted through day 7. In contrast, no gp120 could be detected associated with the cells over the entire 7-day period. However, gp120 protein did accumulate in the medium over the 7-day period. In addition, this protein selectively bound to soluble CD4 (14), and the complex could be specifically immunoprecipitated (data not shown). These results indicate that at least some of the gp160 produced by the *Drosophila* cells is processed to yield an appropriately folded gp120 molecule which does not remain cell associated.

DISCUSSION

The results described here demonstrate that expression of recombinant gp160 in *D. melanogaster* cells is Rev dependent. Expression of envelope protein from a vector encoding gp160 was observed only when Rev protein was supplied in

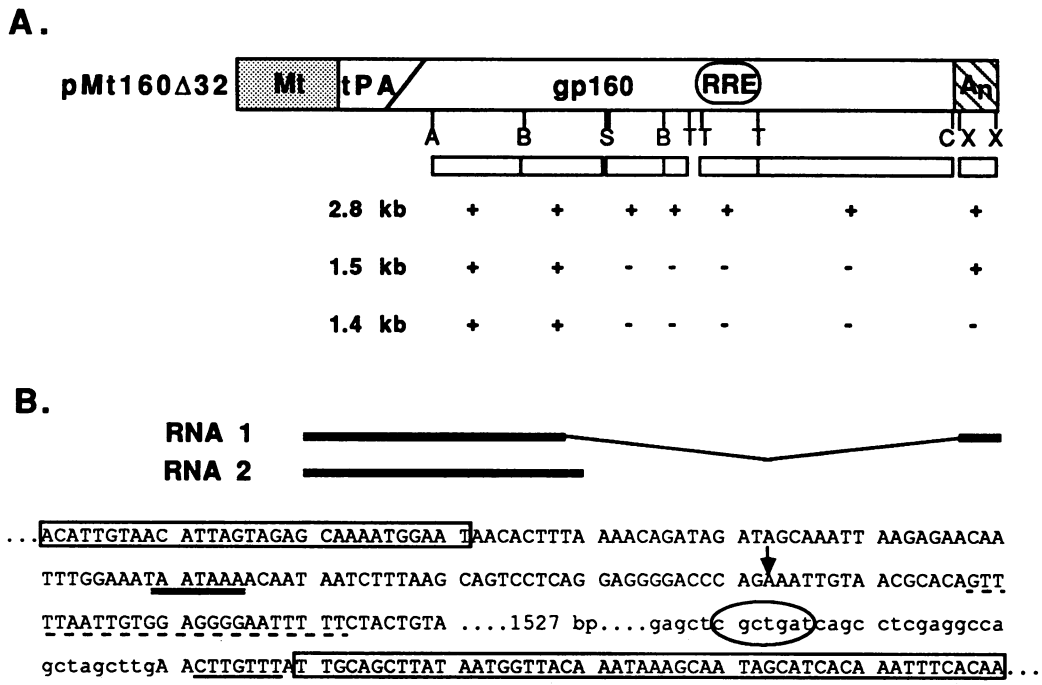

FIG. 5. Analysis of the 1.4- to 1.5-kb *env*-derived RNAs. (A) Diagram of pMt160Δ32 and hybridization pattern of *env*-derived RNAs. Symbols are as in Fig. 1. Restriction enzyme sites used to generate probes for Northern blot analysis are as follows: A, *Apa*LI; B, *Bgl*II; S, *Sca*I; T, *Sty*I; C, *Sac*I; X, *Xho*I. The fragments used as probes are represented as open boxes. The ability or inability of each of the probes to detect the full-length gp160 RNA (2.8 kb) and the smaller *env*-derived RNAs (1.4 and 1.5 kb) in Northern blot analyses is represented + and −, respectively. (B) Structure of *env*-derived RNAs delineated by PCR analyses. An RNA corresponding to the spliced sequence detected (RNA 1) is depicted, with thick lines representing exons and the thin lines representing an intron. An RNA corresponding to the prematurely terminated sequence is depicted by RNA 2. A partial sequence of pMt160Δ32 in the regions where processing occurs is also shown, beginning within the 362-bp *Bgl*II-*Sca*I envelope fragment (capital letters). The number of base pairs of envelope not depicted downstream is shown, followed by a linker sequence (lowercase letters) which connects envelope sequences with SV40 sequences (capital letters). The spliced sequence detected (RNA 1) connects the two sequences shown in boxes. The consensus sequences normally found at splice donor and acceptor sites (43) are not apparent at these junctions. However, a perfect match to a consensus branch point sequence (circled) and a pyrimidine-rich stretch (single underline) are positioned appropriately upstream of the splice acceptor site (25, 39). The position at which the truncated sequence (RNA 2) terminates is shown by an arrow. A consensus polyadenylation signal (AATAAA, double underline) and several GT-rich regions (dashed underline) are located appropriately near the termination site.

trans. Furthermore, gp160 production increased with increasing levels of Rev protein in the cells. Analysis of total RNA demonstrated that synthesis of gp160 message was dependent on induction of the *Drosophila* Mt promoter and

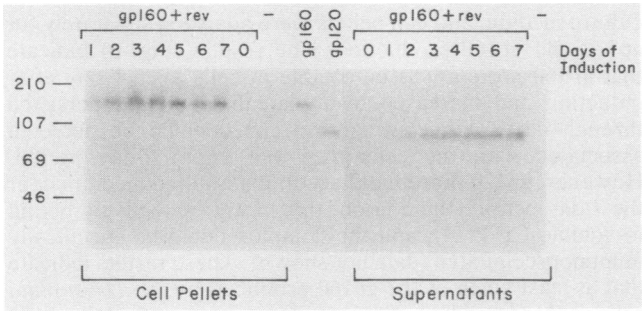

FIG. 6. Western blot analysis of envelope proteins over a 7-day induction. Cells were harvested on the indicated days after induction and analyzed as described for Fig. 2A. Samples shown are from cell line D (gp160 + rev) or from nontransfected cells (−). Whole-cell lysates are shown on the left (cell pellets), and conditioned-medium samples are shown on the right (supernatants). In the middle two lanes, gp160 and gp120 protein standards are shown. Sizes of molecular mass standards are shown on the left (in kilodaltons; see text).

was independent of Rev. However, an analysis of fractionated RNA revealed that full-length, unspliced gp160 mRNA was found in the cytoplasm only in the presence of Rev. In the absence of Rev, this RNA was apparently retained in the nucleus. Thus, Rev appears to regulate envelope expression in *Drosophila* cells by facilitating export of the nucleus-entrapped gp160 mRNA to the cytoplasm. This is the first demonstration of Rev regulation operating in a nonmammalian cell type.

The mechanism by which Rev acts in *Drosophila* cells appears to be similar to that observed previously in mammalian cells (1, 5, 15, 17, 21, 29, 30). Consistent with the results of Felber et al. (17), Malim et al. (30), and Chang and Sharp (5), we found that Rev does not significantly affect the levels of synthesis of its target RNA or the pattern of RNA observed in the cell nucleus; rather, Rev promotes the transport of its target RNA from the nucleus to the cytoplasm. This suggests that recombinant Rev protein synthesized in *Drosophila* cells exerts the same function as mammalian Rev. Most important, any cellular factors involved in mediating the Rev response must be conserved between *Drosophila* and mammalian cells. This is in contrast to what has been observed for the HIV regulatory protein Tat, which functions only poorly, if at all, in *Drosophila* cells (33).

There have been various speculations about how Rev

effects cytoplasmic accumulation of RNAs containing the RRE (1, 5, 15, 17, 21, 29, 30). One possibility is that Rev is involved directly in placing mRNAs into a transport pathway not normally accessed by certain partially or suboptimally spliced messages. Alternatively, Rev may rescue incompletely spliced RNAs from spliceosome complexes that preclude efficient RNA transport. On the basis of the effects of splice site mutations in a heterologous RNA containing the RRE, Chang and Sharp (5) have suggested that splicing signals are involved in the Rev response and that the splicing efficiency of Rev-responsive RNAs must be suboptimal in order to allow Rev to act. When Rev responsiveness is retained even though splicing is eliminated by removing a donor or acceptor signal (15, 20, 26, 30), it has been argued that these RNAs retain some remnant of spliceosome recognition information (5) and are therefore subject to nuclear sequestration and Rev control.

In the case of our *Drosophila* gp160 expression unit, the splice donor and acceptor sequences normally used by HIV for envelope RNA expression have been removed, as have all upstream sequences, including the envelope leader and signal sequence and the splice donor sequence for the *tat* and *rev* RNAs. However, the transcription unit does contain the splice acceptor for the *tat* and *rev* RNAs, which is located within the portion of *env* encoding gp41 downstream from the RRE. Also present in *env* are a number of "cryptic" donor and acceptor sites, such as those used to generate RNA encoding the chimeric protein TEV, which shares sequences with the Tat, Rev, and envelope proteins (3). We found no evidence for the utilization of these sites in the *Drosophila* system. Instead, our data demonstrate the existence of other envelope-derived processed RNA species.

Northern blot and PCR analyses (Fig. 5) revealed that at least one of these RNAs appears to represent a cryptically spliced *env* RNA which is spliced from within the envelope region (position 6819 [36]) into downstream SV40 sequences used for supplying polyadenylation information to the transcription unit. The detection of this cryptic splice site and the occurrence of other known splice information on our transcription unit (whether utilized or not) are consistent with the possibility that spliceosome recognition and/or suboptimal splicing may be required to achieve Rev-regulated function. In this regard, it is important to point out that we have observed efficient Rev-independent gp120 envelope expression in *Drosophila* cells from a construct essentially analogous to that described above except that the gp41 coding sequences were deleted (Ivey-Hoyle et al., in press). This gp120 expression vector also encoded cryptic splice information, yet it expressed gp120 in the absence of Rev. This observation suggests that sequences in the gp41 coding region must be playing a role in the nuclear retention of the gp160 RNA. The fact that the smaller processed *env*-derived RNAs that we observed also lacked the gp41 coding region and appeared to escape nuclear retention is consistent with this idea.

What elements in the gp41 coding region are involved in the nuclear retention of messages? The RRE is located in the gp41 coding region, but recombinant DNA studies have shown that the mere presence of the RRE on an RNA does not result in selective nuclear retention (5, 15). In contrast, the presence of sequences from the gp41 coding region which encompass the *tat* and *rev* splice acceptor has been shown to restrict RNA to the nucleus (15). Hence, it may well be that the Tat and Rev splice acceptor is involved in the selective nuclear retention of RNAs under normal Rev control.

One of the processed envelope RNA species observed in our system appears to represent an unspliced, polyadenylated, prematurely terminated RNA which stops within the envelope coding sequence (position 6868 [36]). Examination of the DNA sequence surrounding this site reveals the presence of a consensus AATAAA poly(A) addition site sequence 38 bp upstream and several GT-rich sequences downstream of the polyadenylation point. Whether this site is ever used by HIV is unknown. Its utilization in *Drosophila* cells may result simply from the creation of the chimeric transcription unit used to examine envelope expression; alternatively, the existence of this truncated message may depend upon some subtle difference in the *Drosophila* polyadenylation recognition system relative to its mammalian counterpart. In any case, the occurrence of this message represents an example of the use of an AATAAA signal contained within a coding segment which under a particular set of conditions is exposed for recognition and function.

Although multiple *env*-related RNAs were generated from our gp160 expression vector, only protein products corresponding to the full-length gp160 mRNA were observed. A smaller truncated protein of the size encoded by the 1.4- to 1.5-kb RNAs was not detected, possibly because of rapid turnover or the inability of our polyclonal serum to recognize this portion of the gp120 protein. More important, the gp160 protein expressed in *Drosophila* cells from the full-length RNA remained cell associated and appeared to be cleaved to produce a gp120-sized molecule that rapidly dissociated from the cell and was found to accumulate in the medium. This gp120 protein recognizes and binds to a soluble form of the human CD4 protein (M. Ivey-Hoyle, unpublished) and thus retains its receptor recognition properties. Expression and processing of full-length gp160 in mammalian cells normally results in cell surface expression of envelope protein, with significant levels of gp120 remaining associated with the cells (references 4 and 35 and references therein). Our preliminary data suggest that the dissociation of the *Drosophila*-expressed gp120 molecule appears to occur because it is lacking the N-terminal 31 amino acids of the mature viral protein. Expression of an otherwise identical gp160 construct in which these 31 amino acids have been restored produces a gp120 which remains cell associated.

In conclusion, we have established a novel system with stably transfected *Drosophila* Schneider 2 cells which reproduces the regulation of HIV-1 envelope expression by the viral Rev protein. Our data indicate that the mechanism of Rev action is conserved in these cells. The system allows us to use metal-regulated promoter control to selectively turn on efficient Rev-regulated expression of gp160. This system should be useful in further characterizing the potential role of splicing and, most important, the involvement of cellular factors in mediating the Rev response.

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